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Characterization of the Cellulose-Binding Domain of the *Clostridium cellulovorans* Cellulose-Binding Protein A

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Cellulose-binding protein A (CbpA), a component of the cellulase complex of *Clostridium cellulovorans*, contains a unique sequence which has been demonstrated to be a cellulose-binding domain (CBD). The DNA coding for this putative CBD was subcloned into pET-8c, an *Escherichia coli* expression vector. The protein produced under the direction of the recombinant plasmid, pET-CBD, had a high affinity for crystalline cellulose. Affinity-purified CBD protein was used in equilibrium binding experiments to characterize the interaction of the protein with various polysaccharides. It was found that the binding capacity of highly crystalline cellulose samples (e.g., cotton) was greater than that of samples of low crystallinity (e.g., fibrous cellulose). At saturating CBD concentration, about 6.4 μmol of protein was bound by 1 g of cotton. Under the same conditions, fibrous cellulose bound only 0.2 μmol of CBD per g. The measured dissociation constant was in the 1 μM range for all cellulose samples. The results suggest that the CBD binds specifically to crystalline cellulose. Chitin, which has a crystal structure similar to that of cellulose, also was bound by the CBD. The presence of high levels of cellobiose or carboxymethyl cellulose in the assay mixture had no effect on the binding of CBD protein to crystalline cellulose. This result suggests that the CBD recognition site is larger than a simple cellobiose unit or more complex than a repeating cellobiose moiety. This CBD is of particular interest because it is the first CBD from a completely sequenced nonenzymatic protein shown to be an independently functional domain.

Crystalline cellulose is resistant to the action of many β-1,4-glucanases. The closely packed cellulose chains are stabilized by hydrogen bonding to form a tight, regular array which shields many of the glycosidic bonds from enzymatic attack (27). Nevertheless, some *Clostridium* species can degrade crystalline cellulose into simple sugars. Several of these strains produce a cellulase enzyme complex (cellulosome) containing a variety of β-1,4-glucanases together with proteins with no known enzymatic activity (15, 16, 20). A previous study (20) has shown that the association of enzyme subunits and nonenzymatic subunits was essential for the degradation of crystalline cellulose, whereas the dissociated enzyme subunits could degrade only noncrystalline substrates.

We have isolated the cellulosome from *Clostridium cellulovorans*, an organism shown to have cellulolytic activity. This cellulase complex has a rather high specific activity compared with those of other cellulases that have been characterized (20). The purified nonenzymatic subunit protein had a very strong affinity for crystalline cellulose. Therefore, this protein was designated as the cellulose-binding protein A (CbpA). Recently, we cloned the structural gene encoding this protein (*cbpA*) and determined its complete nucleotide sequence (21). The amino acid sequence of CbpA deduced from the nucleotide sequence (1,848 amino acid residues, 189 kDa) revealed the following interesting features: (i) a putative cellulose-binding domain (CBD) (amino acids 27 to 189) which was homologous to several known cellulose-binding sequences (10, 21), (ii) hydrophilic regions repeated four times, and (iii) hydrophobic regions repeated eight times. It has been shown that the ability to degrade crystalline substrates is correlated with the binding ability of the cellulase (14). Presumably the CBD mediates the interaction of the nonenzymatic protein, CbpA, with cellulose. The cellulose is then degraded by the cellulosomal enzymatic components, particularly endoglucanases, which are bound to CbpA. In this way CbpA directs the enzymatic components to the crystalline cellulose surface.

This paper describes (i) the subcloning of the region encoding the putative CBD from CbpA and (ii) the interaction between crystalline cellulose and CbpA. A previous study (9) had suggested that the surface of the cellulose crystal presented a regular, repeating set of overlapping potential binding sites and that adsorption of a CBD to this surface could not be analyzed by a classical equilibrium partition analysis. However, we have established experimental conditions which minimize nonspecific binding to the assay tubes and to the cellulose, thereby allowing us to analyze the interaction in terms of a reversible, two-component equilibrium binding system (19). Therefore we were able to determine both the dissociation constant of the cellulose-CBD complex and the binding capacity of the cellulose sample by means of double-reciprocal plots. The substrate specificity of the CBD and the effects of varied pH and soluble carbohydrates on CBD binding to cellulose were also investigated.

MATERIALS AND METHODS

Bacterial strains and plasmids. *Escherichia coli* XL1-Blue was obtained from StrataGene, La Jolla, Calif., and was used for all cloning experiments. *E. coli* BL21 (DE3) and pET-8c were as described previously (23).

Materials. PC buffer (pH 7) contained 50 mM KH_2PO_4, 10 mM sodium citrate, and 1 mM NaN_3. TEDG buffer (4) contained 10 mM Tris (pH 7), 0.1 mM EDTA, 0.1 mM

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dithiothreitol, and 5% (vol/vol) glycerol. Although Tris has a low buffer capacity at pH 7, the buffer was suitable because hydrogen ions were neither produced nor used. Restriction endonucleases were from Bethesda Research Laboratories, Bethesda, Md. All other chemicals used were of the highest purity commercially available. Avicel PH101 (lot 1117) was from FMC Corp., Philadelphia, Pa. Absorbent cotton was from the Seamless Rubber Co., New Haven, Conn. Cellulon fiber was from Weyerhaeuser, Tacoma, Wash. Granular chitin from crab shells was a kind gift of Demosthenes Pappagianis. All other binding substrates were purchased from Sigma Chemical Co., St. Louis, Mo. Each of the polysaccharides was washed twice with PC buffer before use. Nigeran was recrystallized by dissolving the solid in hot water, filtering, and cooling on ice. The fiber sizes of the cellulon and cotton were reduced by processing with a Gifford-Wood minimill for 5 min.

Cloning of putative CBD. DNA primers complementary to the regions of *cbpA* flanking the putative CBD (CbpA residues 28 to 189) were synthesized by a Gene Assembler Plus (Pharmacia). The forward primer contained an *Nco*I restriction site (recognition sequence, CCATGG) with the ATG in frame with the gene fragment to act as a translational start codon when cloned into the pET-8c vector cloning site. The reverse primer contained a stop codon and a *Bam*HI site. Polymerase chain reaction (PCR) amplification was performed with 20 pmol of each primer, 200 μM each deoxynucleoside triphosphate, and 1 ng of *cbpA* DNA (cloned into vector pGEMEX-1 [Promega] as in reference 21) as a template in a total volume of 100 μl. *Taq* polymerase was obtained from Amersham and was used under buffer conditions recommended by the manufacturer. PCR was carried out for 40 cycles as described previously (11). The PCR product was purified by phenol-chloroform extraction followed by ethanol precipitation and a wash with 70% ethanol; it was then dried under vacuum and resuspended in 27 μl of distilled water. The DNA was then cleaved with *Nco*I and *Bam*HI and run on a 2.5% low-melting-point agarose (Nuseieve GTG; FMC Corp.) gel in TBE buffer (18). DNA bands stained with ethidium bromide were visualized under long-wave UV light and cut from the gel. The vector, plasmid pET-8c, was prepared by cleaving 1 μg of pET-8c DNA with *Nco*I-*Bam*HI and cutting the linearized DNA band from the gel. Vector and insert DNAs were ligated by using 100 ng of vector DNA and 300 ng of insert with a Takara Ligation kit. The ligated plasmids were used to transform competent *E. coli* XL1-Blue cells, which were then plated on Luria-Bertani (LB) plates (18) containing 100 μg of ampicillin per ml and 12.5 μg of tetracycline per ml. After overnight incubation at 37°C, colonies were selected and grown in liquid LB medium containing ampicillin and tetracycline. Plasmid DNA from each culture was rescued as described previously (23) and cleaved with restriction enzymes to verify the insertion of the gene fragment. The insert sequence was confirmed by DNA sequencing, using the same procedures as reported previously (21).

Preparation and purification of CBD protein. Plasmid DNA containing the insert was used to transform *E. coli* BL21 (DE3). Plasmid-containing cultures were grown at 37°C with shaking in NZCYM (18) medium containing ampicillin (100 μg/ml) to a Klett reading of 160 (green filter). At this point, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM. After 4 h, the cells were harvested by centrifugation, resuspended in PC buffer containing RNase A at 10 μg/ml and DNase I at 1 μg/ml, and lysed by sonication on ice with a Biosonic II sonicator at maximum power for 45 s followed by a 15-s cooling period. This was repeated four times. The insoluble fraction of a 1-liter cell culture was collected by centrifugation (30 min at 12,000 × *g* and 4°C) and resuspended in 20 ml of 6 M guanidine HCl. This was kept on ice for 30 min with occasional vortexing to disperse the pellet. Insoluble debris were removed by centrifugation (30 min at 12,000 × *g* and 4°C). The soluble guanidine HCl extract was gradually diluted to a total volume of 400 ml with TEDG renaturation buffer over a 2-h period at 4°C. Ammonium sulfate was added to 80% saturation. After 4 h at 4°C, precipitated proteins were collected by centrifugation (30 min at 12,000 × *g* and 4°C), resuspended in 40 ml of PC buffer, and dialyzed against PC buffer. The CBD protein fragment of CbpA was further purified by cellulose affinity, as follows. Three additions of 1.0 g of Avicel PH101 microcrystalline cellulose were used to remove the CBD protein from the solution. After each addition, the suspension was allowed to come to equilibrium (1 h at room temperature with slow rotation). The cellulose was then collected by centrifugation and removed before the next addition. The 3 g of cellulose was washed once with 1 M NaCl–PC buffer and twice with PC buffer. Purified CBD was eluted from the cellulose by three washes with 10 ml of 6 M urea. The urea fractions were pooled and dialyzed against PC buffer. The protein concentration in the final purified fraction was analyzed by colorimetric methods, using the MicroBCA protein assay kit (Pierce, Rockford, Ill.) with bovine serum albumin (BSA) standards.

Determination of the CBD-cellulose dissociation constant and the cellulose-binding capacity. Samples of CBD protein (typically 0 to 100 μg) were added to 1.5-ml-capacity microcentrifuge tubes containing PC buffer supplemented with 1 mg of BSA per ml and the desired amount of cellulose (typically 1 mg added from a stock slurry containing 10 mg of cellulose per ml and 1 mg of BSA per ml in PC buffer). Potential competitors, e.g., cellobiose (4 mg/ml) or carboxymethyl cellulose (CMC) (4 mg/ml), were included in some reactions by adding 200 μl of a 20-mg/ml stock solution in PC-BSA buffer. The final volume was always 1 ml. The pH of the buffer was 7.0 except when noted otherwise. For experiments at other pH values, the PC-BSA buffer was adjusted prior to use by the addition of concentrated HCl or NaOH. Assay tubes were mixed by slow vertical rotation (30 rpm) at 37°C for 1 h. The samples were then spun in a microcentrifuge for 1 min to sediment the cellulose and cellulose-protein complexes. After the buffer had been removed, the pellet was washed by resuspension in 1 ml of PC buffer. The wash was separated out by centrifugation and discarded. Pellets were then resuspended in a final 1 ml of PC buffer. (The centrifugation step would not be expected to perturb the equilibrium because the cellulose and protein-cellulose were concentrated to the same extent.) Of the original BSA in the assay tubes (~1 mg/ml), only about 0.1 μg would remain after the washing steps, assuming no nonspecific adsorption and a liquid volume of 10 μl in the pellet. Any color development as a result of this residual BSA was controlled for by the 0 CBD control tubes. Aliquots (150 μl) of this well-mixed suspension were taken for protein determinations with the MicroBCA kit. The manufacturer's instructions were followed, except that the sample volume was brought to 0.5 ml with PC buffer, to which 0.5 ml of BCA working reagent (Pierce Co.) was added. Assay mixtures were incubated at 60°C for 30 min. The protein concentration was determined colorimetrically from the cleared supernatants at 562 nm in a Shimadzu 160 U spec-

trophotometer. Assay tubes to which no CBD protein was added were used to correct for a small amount of color development caused by the cellulose and residual BSA. The data were compared with BSA standards and adjusted to accommodate the dilutions that were made to determine the amount of protein bound to the cellulose in each sample. The practical detection limit of this assay was about 0.2 μg/ml. After correction for dilutions, this corresponds to about 0.034 nmol of CBD bound to the cellulose in the assay tube. The free CBD protein concentration, [P], was determined by subtracting the bound protein concentration, [PC], from the total CBD added to the tube, $[P]_t$:

$$[P] = [P]_t - [PC] \quad (1)$$

The system was analyzed by assuming a simple equilibrium interaction (19):

$$P + C \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} PC$$

where the dissociation constant, K_d, is defined as

$$K_d = \frac{k_{-1}}{k_1} = \frac{[P][C]}{[PC]}$$

The data were analyzed by double-reciprocol plots of 1/[PC] versus 1/[P] at different fixed levels of cellulose (equation 2):

$$\frac{1}{[PC]} = \frac{K_d}{[PC]_{max}} \times \frac{1}{[P]} + \frac{1}{[PC]_{max}} \quad (2)$$

It must be noted that the cellulose is not a soluble component, so that [C] represents the concentration of binding sites on the cellulose surface exposed to the buffer, per unit volume. Similarly, [PC] represents the concentration of binding site-protein complexes per unit volume. Straight lines were fitted to the datum points by the least-squares method, using the DeltaGraph Professional plotting application (Deltapoint, Inc., Monterey, Calif.). Each point was the average of three independent protein assays from the same binding-assay tube. Experiments were performed in duplicate. At least two different amounts of cellulose were used to determine the K_d and $[PC]_{max}$/g of cellulose. These were averaged to provide the values listed in Table 1.

Determination of binding to other polysaccharides. Xylan, nigeran, Sephadex G-75, and chitin were used in assays to determine whether they were substrates for CBD protein. In all cases, the methods used were the same as those used in determining the binding to cellulose. Chitin exhibited a very high background in the MicroBCA assay, which increased proportionally to the incubation time at 60°C, and so the color development time was reduced to 15 min. Because of the high background of chitin, only two widely different protein concentrations were used.

RESULTS

Purification of the CBD for binding analyses. To selectively produce the putative CBD region of CbpA, residues 28 to 189, we designed oligonucleotide primers complementary to bases 67 to 86 and 558 to 579 of *cbpA* (Fig. 1). As shown in Fig. 1, these primers were designed with mismatches to create an *Nco*I site and an ATG start codon on one end of the PCR product and a TAG stop codon followed by a *Bam*HI site at the other end. This gene fragment was then cloned into the T7 RNA polymerase expression plasmid pET-8c, resulting in plasmid pET-CBD. The cloned gene fragment codes for a methionine at the N terminus of the CBD, but the rest of the CBD amino acid sequence corresponds to residues 28 to 189 of CbpA. The protein fragment has a molecular weight of 17,634. The insertion was verified by DNA sequencing. CBD protein was produced by *E. coli* BL21 (DE3) cells harboring pET-CBD. After the addition of IPTG, this host strain produces T7 RNA polymerase, which recognizes the T7 promoter in the pET vector. The *cbd* gene fragment was under the control of this inducible promoter, and CBD protein was synthesized in large amounts after induction (Fig. 2). After a 4-h production period, the soluble extract from the lysed cells contained only small amounts of CBD protein, while most was found in the insoluble fraction. This protein was readily soluble in concentrated guanidine hydrochloride and was renatured by slow dilution into TEDG buffer. It was found that protein prepared in this fashion binds to Avicel, verifying the putative CBD. Although this fraction is mostly CBD protein, the assays described here require the protein to be very pure. This purity is obtained by a single cellulose affinity step, as described in Materials and Methods. The affinity-purified CBD protein appears on acrylamide gels as a single band when stained with Coomassie brilliant blue. Approximately 70 mg of CBD protein can be recovered from the cells harvested from a 1-liter culture.

TABLE 1. Adsorption of CBD protein to insoluble substrates

Substrate	Observed K_d (μM)[a]	Observed $[PC]_{max}$ (μmol of CBD/g)[a]
Avicel PH101	0.6	2.1
Sigmacell 20	1.1	1.2
Sigmacell 50	1.4	1.7
Sigmacell 100	1.3	0.5
Microgranular cellulose	1.0	0.4
Fibrous cellulose	1.4	0.2
Cotton	0.8	6.4
Cellulon	1.2	5.3
Xylan		0
Sephadex G-75		0
Nigeran		0
Chitin	1.0	1.6
Avicel PH101[b]		
+CMC (4 mg/ml)	0.9	1.9
+Cellobiose (4 mg/ml)	0.5	1.8

[a] The values for K_d and $[PC]_{max}$ were calculated as described in Materials and Methods.

[b] Competition experiments including CMC or cellobiose were performed with Avicel PH101 at 1 mg/ml. The values given for the competition experiments represent the dissociation constant and binding capacity of the CBD-Avicel pair.

Time course of binding of CBD to cellulose. The time course of the interaction of Avicel with CBD (Fig. 3) discloses several features of the process. (i) At initial concentrations of 1.0 mg of Avicel per ml and 2.0 μM CBD (i.e., $[P]_0$), a plateau value of 1.2 μM complex (i.e., [PC]) is attained by 60 min. A separate experiment established that the maximum CBD-binding capacity of the cellulose sample was 2.1 μmol g^{-1}, corresponding to an effective concentration of 2.1 μM total cellulose sites (i.e., $[C]_0$). Assuming that an equilibrium was established (verified below), K_d, defined as [P][C]/[PC], is about 0.6 μM. (ii) The second-order rate constant for

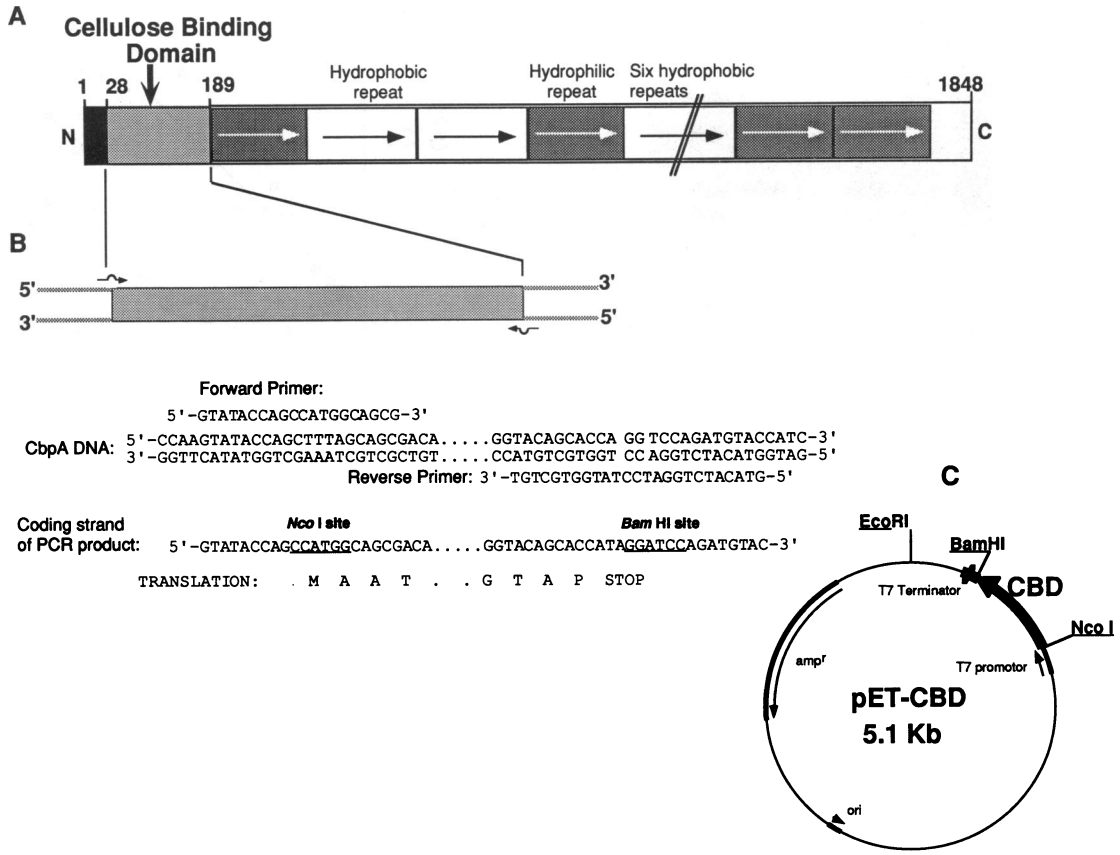

FIG. 1. Preparation and cloning of the CBD gene fragment. (A) Analysis of the primary structure of CbpA, which contains an N-terminal signal peptide, a unique CBD region, four hydrophilic repeats (white arrows), and eight hydrophobic repeats (black arrows). (B) PCR primer placement along the *cbpA* gene. Included for clarity are the primer sequences and the *cbpA* DNA sequence of the CBD flanking regions. The PCR product contains *Nco*I and *Bam*HI sites (underlined). Also note that the ATG start codon for the gene fragment is located within the *Nco*I site and the TAG stop codon is adjacent to the *Bam*HI site. (C) Schematic of pET-CBD, containing the CBD gene fragment cloned into the pET-8c vector. The vector contains the necessary transcriptional and translational signals for inducible CBD production.

association (k_1) calculated from the integrated rate equation for a reversible Bi Uni reaction (3, 26) is about 2.7×10^4 M^{-1} min^{-1} (average value for points from 5 to 60 min). The rate constant for the dissociation of the complex (k_{-1}), calculated as k_1K_d, was 1.6×10^{-2} min^{-1} ($t_{1/2}$ = 43 min). The relatively long $t_{1/2}$ for complex dissociation permitted the C+PC pellet to be washed once without significant loss of bound CBD. (Resuspension and recentrifugation of the initial pellet were completed in less than 1 min. During this period, less than 3% of the bound CBD would be lost.) It was also observed that after prolonged incubation, the measured [PC] declined, dropping to about 50% of the maximum value after 18 h. This may be caused by gradual denaturation of the protein or by disruption of the cellulose surface by nonhydrolytic processes as described by Din et al. (7). To reduce artifacts resulting from these effects, we used the shortest incubation time for which equilibration appeared to be "complete." (Any further increase in binding beyond 60 min would be obscured by the experimental error.)

Analysis of the CBD cellulose-binding affinity and binding capacity. Figure 4 shows a typical diagnostic plot of the binding of pure CBD to Avicel microcrystalline cellulose. Within experimental error the plots were linear, yielding a K_d of about 0.6 μM and a $[PC]_{max}$ of 2.1 μmol of CBD bound per g of Avicel. The latter value corresponds to approximately 37 mg of CBD protein per g of Avicel. The linearity of the diagnostic plots suggests that only one type of CBD-cellulose interaction is occurring.

The ability of CBD to bind cellulose types other than Avicel was also investigated. Table 1 shows the values for the K_d and $[PC]_{max}$ found for each of the substrates. Sigmacell 20 and 50 are described as microcrystalline forms of cellulose, and these also bound to CBD. Highly crystalline forms of cellulose such as absorbent cotton and Cellulon fiber (crystalline cellulose from *Acetobacter xylinum*) were able to bind substantially more of the CBD (up to 6.4 μmol of CBD per g of substrate). Fibrous and microgranular cellulose, however, which are more highly processed and thus contain less of the native crystalline form, bound a smaller amount of the CBD. The K_d for CBD-cellulose was about the same for all forms of cellulose, whereas the $[PC]_{max}$ varied over a 30-fold range.

Binding-site competition. To determine whether soluble carbohydrates competed with Avicel for the CBD protein, cellobiose (a β-1,4-linked glucose dimer) and CMC (a soluble carboxymethyl derivative of cellulose) were included in some assays at four times the weight/volume of Avicel (1 mg of Avicel and 4 mg of cellobiose or CMC per ml of assay). No large differences in the K_d or $[PC]_{max}$ were observed

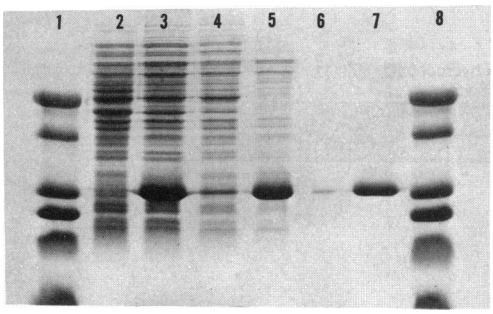

FIG. 2. Expression and purification of the CBD protein. Whole-cell proteins from cells harboring pET-8c (lane 2), whole-cell proteins from cells harboring pET-CBD (lane 3), cytosolic fraction from lysed pET-CBD cells (lane 4), guanidine HCl-solubilized membrane/inclusion body fraction from lysed pET-CBD cells (lane 5), final PC buffer wash of Avicel pellet (lane 6), and purified CBD protein (lane 7) were loaded on a 15% acrylamide gel. Each lane was loaded with 0.005% of the total protein of each fraction, except lane 6, which is a 10× concentrate. Prestained molecular mass markers (lanes 1 and 8) have mobilities of approximately 2.6, 5, 12.7, 18.1, 29, and 44 kDa.

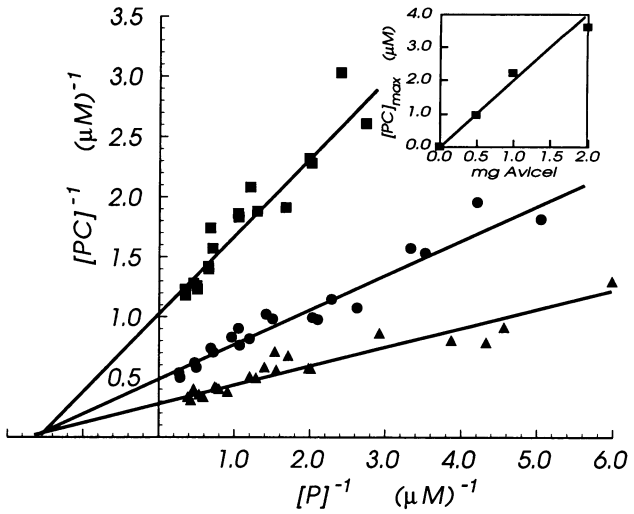

FIG. 4. Double-reciprocal plot of CBD binding to Avicel. Symbols: ■ 0.5 mg of Avicel; ●, 1 mg of Avicel; ▲, 2 mg of Avicel. The inset shows $[PC]_{max}$ plotted against the amount of Avicel used. The assay volume was 1.0 ml.

(Table 1), indicating that these soluble carbohydrates had little or no effect on the binding of the CBD to Avicel.

Effect of pH on dissociation constant. *C. cellulovorans* is a neutrophilic organism, thriving only around pH 7 (22), so this pH was used for most of the binding assays. However, other experiments established that the K_d and $[PC]_{max}$ did not vary significantly with changes in pH over the range 5.0 to 8.0. In addition, it was noted that PC buffers as acidic as pH 3.5 or as basic as pH 9.5 would not remove the CBD from Avicel during 1-min washes (data not shown).

Binding of the CBD to other polysaccharides. Xylan, Sephadex G-75, nigeran, and chitin were used to explore the binding specificity of the CBD. Of these, only chitin showed measurable binding of the CBD peptide (Table 1). The chitin-CBD K_d and binding capacity were similar to the Avicel-CBD values.

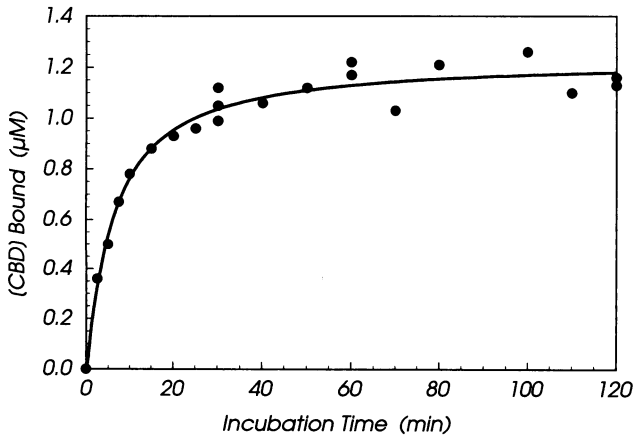

FIG. 3. Time course of CBD-Avicel binding. CBD (total protein, 2.0 μM) and Avicel (1 mg/ml) were equilibrated as described in Materials and Methods, except that a larger total volume was used to provide samples taken at various time points. Each time point sample was washed and assayed as described in Materials and Methods.

DISCUSSION

Many cellulases and other hydrolytic enzymes, such as chitinases, have high affinities for their substrates (2, 20). Much interest has recently focused on identifying individual domains of these enzymes that are responsible for the binding (8). Several bacterial cellulases take the idea of a binding domain one step further and produce a cellulase-related protein that is nonenzymatic but mediates the interaction between the endoglucanases and the insoluble substrate (15, 20, 21). It has previously been shown that the ability of a cellulase to degrade crystalline cellulose is related to the strength of the binding between the cellulase and crystalline cellulose (14), whereas strong binding is not related to the ability to degrade amorphous cellulose.

Our results show that CbpA contains a domain responsible for cellulose binding and that this domain could function independently from the rest of CbpA. Because the purification protocol involved denaturation and renaturation steps, the fact that the purified protein was functional indicates that the CBD protein sequence was sufficient for proper folding of the protein fragment.

Previous attempts to determine the relative binding affinity of cellulases for the surface of cellulose have been made. As reviewed by Klyosov (14), the basic concept involved determining a partition coefficient (K_p) between the insoluble cellulose and the soluble phase (i.e., moles protein per gram of cellulose/concentration of protein in the aqueous phase), which was typically expressed in units of liters per gram. These partition coefficients were used as a general estimation of the relative binding ability of the cellulase. More recent attempts to derive a more traditional measurement, using an actual affinity constant, K_a, have yielded nonlinear diagnostic plots (9). The authors suggested that the nonlinearity was a result of mutually hindered binding (negative cooperativity) because the cellulose surface is a two-dimensional array of overlapping potential binding sites.

There are several alternative explanations for the nonlinearity of diagnostic plots reported in earlier work. (i) The first is nonspecific binding of the protein to the assay tube and perhaps also to the surface of the cellulose. These

problems are compounded when the free protein rather than the bound protein is used for the measurements. When the free-protein concentration is very low, the relative amount lost due to tube interactions is large; when the free-protein concentration is high enough to minimize the effects of such nonspecific binding, the experimental error involved in the protein determination itself may mask the relatively small changes in free-protein concentration being measured. (ii) The simultaneous weak binding of catalytic sites to amorphous cellulose can affect the linearity of diagnostic plots for CBD-containing cellulases. (iii) A cellulase or CBD may have different affinities for different crystal faces of crystalline cellulose. As an example, it was suggested that the CBD from CenA binds preferentially to the 110 face of cellulose crystals but that the $1\bar{1}0$ face, which has a higher binding capacity (but apparently lower affinity), is filled only at high ligand concentration (9).

We have found nonspecific binding of the CBD to the assay tubes to be a problem in performing equilibrium binding experiments, and we have developed an assay in which the CBD and cellulose are equilibrated in the presence of excess BSA. The BSA effectively eliminates nonspecific CBD interactions with the tube. After equilibrium is reached, the cellulose and cellulose-protein complexes are collected, washed, and assayed for bound proteins. As described above the dissociation of the CBD-cellulose is slow so that no detectable amount is removed during a rapid wash step.

The bound CBD concentration was measured directly by the protein assay, and the free CBD concentration was calculated by subtracting the bound CBD concentration from the total CBD concentration, as shown in equation 1. This has the advantage that any CBD molecules adsorbed nonspecifically with low affinity to the cellulose would be removed by the wash step, resulting in data that more accurately reflect the specific, high-affinity interaction between the CBD and the cellulose surface. As shown in Fig. 4, data gathered by using this type of assay yields (within experimental error) linear diagnostic plots. The validity of the assay is supported by the observation that $[PC]_{max}$ increases linearly with the amount of cellulose used, whereas K_d is independent of the amount of cellulose. Table 1 shows the results obtained with several forms of cellulose as well as with other carbohydrates. The results indicate that cellulose types described as crystalline have a higher CBD-binding capacity than do highly processed celluloses that have lost much of their crystallinity. The fact that the $[PC]_{max}$ of cellulose samples varies widely with different cellulose types whereas the K_d remains constant indicates that we have measured one type of strong protein-cellulose interaction occurring between the CBD and the cellulose. The lower $[PC]_{max}$ of highly processed celluloses reflects a smaller number of potential protein interaction sites in the sample and seems to correlate with the crystallinity of the sample. This would indicate that there is some special feature present in crystalline cellulose that makes it acceptable as a binding substrate, whereas amorphous cellulose is found lacking.

To further characterize the substrate specificity of the CBD, we measured the effect of added soluble substrates (cellobiose or CMC) on cellulose binding. Excess cellobiose or CMC had no effect on the CBD-Avicel K_d or $[PC]_{max}$, as shown in Table 1. This lack of competition confirms that the CBD recognition site is specific for something more complex than a simple repeating glucose or cellobiose moiety and suggests that a particular three-dimensional arrangement of cellulose chains is needed.

The specificity of the CBD for crystalline cellulose prompts a consideration of chitinases, which are known to bind tightly to chitin, a polymer of *N*-acetylglucosamine in β-1,4 linkage. Like cellulose, chitin comes in a variety of forms, depending on the source and the purification method used in its isolation (1, 2). The chitin used for affinity purification of chitinases is α-chitin, in which the chains are arranged in an antiparallel fashion. This form of chitin is crystalline with a structure similar to that of native crystalline cellulose (often referred to as cellulose I). Cellulose I is the form in which the cellulose chains are arranged in parallel bundles, as opposed to cellulose II, in which the chains are in an antiparallel configuration. Processing of cellulose I under harsh conditions causes its disruption, resulting in cellulose II (25). Both forms are crystalline, because of extensive hydrogen bond formation. Since our isolated CBD binds to less highly processed forms of cellulose, i.e., largely cellulose I, we were interested in finding whether the CBD would bind to α-chitin, which has a similar crystal structure, although of opposite strand orientation. We found that the CBD did accept chitin as a binding substrate with a K_d very similar to that for cellulose. Chitin is the only noncellulosic substrate that we have found that is able to bind the CBD. Xylan (β-1,4-xylose), nigeran (alternating α-1,4- and α-1,3-glucose), and Sephadex G-75 (α-1,6-glucose with α-1,3 branches) (5) were also tried, but the CBD did not show measurable binding to any of them under the conditions of the assay. Since chitin is the only one of these substrates that is crystalline, we believe that this demonstrates the importance of crystallinity in the substrate. It is possible that the CBD is specific for a long, rigid carbohydrate chain or recognizes an area of adjacent chains with bond distances similar in both chitin and cellulose.

A parallel can be drawn between endo-β-1,4-glucanases and xylanases. Neither type of enzyme has been reported as binding tightly to its noncrystalline substrate (amorphous cellulose and xylan, respectively). No xylan-binding domains have been reported, but some xylanases, like some endo-β-1,4-glucanases, have CBDs specific for crystalline cellulose (12). Although it seems illogical for an enzyme to have a high affinity for a substrate it cannot hydrolyze, on further consideration, taking into account the proximity of xylan and cellulose in native cellulosic materials, this fact suggests that crystalline cellulose makes a better anchoring site for glycan hydrolases than does xylan or amorphous cellulose.

To make use of the dissociation constant that we have determined, we must compare the CBD-cellulose binding with that in other systems previously studied. However, earlier work on cellulases is reported mostly in the form of partition coefficients (e.g., the work of Klyosov [13]). Our data can be converted to this form, yielding a result of K_p = 1.5 liters/g. This places the tightness of the CBD-Avicel binding fairly near the top of the logarithmic range (systems with the weakest binding have $K_p < 0.004$, and those with the strongest binding have $K_p = 8$). It must be noted that differences in the experimental procedures may reduce the significance of this direct comparison, because of the problems described above.

Comparisons with systems for which true K_d values have been published are likely to hold more meaning. The K_ds of proteins for various soluble carbohydrates have been determined. As reported by Szmelcman et al. (24), the *E. coli* maltose-binding protein binds maltose with a K_d of 1 μM and

maltotriose binds with a K_d of 0.16 μM. Similarly, the *E. coli* xylose-binding protein binds xylose with a K_d of 0.63 μM (6). Therefore, our finding of about 0.6 μM for the Avicel-CBD K_d is well within the reported range for carbohydrate-protein interactions.

A qualitative analysis of a CBD of the cellulosome subunit S1 from *C. thermocellum* YS has been reported (17). The CBD of *C. cellulovorans* CbpA has approximately 50% homology with the CBD region reported for *C. thermocellum*. The CBD region of *C. cellulovorans* CbpA is unique (21), whereas the number of CBD regions in *C. thermocellum* is unknown, since the complete sequence of subunit S1 has not been reported.

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REFERENCES

1. **Blackwell, J.** 1988. Physical methods for the determination of chitin structure and conformation. Methods Enzymol. **161**:435–442.
2. **Cabib, E.** 1988. Chitinase from *Serratia marcescens*. Methods Enzymol. **161**:460–462.
3. **Capellos, C., and B. H. Bielski.** 1980. Kinetic systems: mathematical description of chemical kinetics in solution, p. 43–45. Robert E. Krieger Publishing Co., Huntington, N.Y.
4. **Chang, B. Y., and R. H. Doi.** 1990. Overproduction, purification, and characterization of *Bacillus subtilis* RNA polymerase sigma A factor. J. Bacteriol. **172**:3257–3263.
5. **Coutinho, J. B., N. R. Gilkes, R. A. J. Warren, D. G. Kilburn, and R. C. Miller.** 1992. The binding of *Cellulomonas fimi* endoglucanase C (CenC) to cellulose and Sephadex is mediated by the N-terminal repeats. Mol. Microbiol. **6**:1243–1252.
6. **Dahms, A. S., W. Huisman, G. Neslund, and C. Ahlem.** 1982. D-Xylose-binding protein (periplasmic) from *Escherichia coli*. Methods Enzymol. **90**:473–476.
7. **Din, N., N. R. Gilkes, B. Tekant, R. C. Miller, R. A. J. Warren, and D. G. Kilburn.** 1991. Non-hydrolytic disruption of cellulose fibres by the binding domain of a bacterial cellulase. Bio/Technology **9**:1096–1099.
8. **Gilkes, N. R., B. Henrissat, D. G. Kilburn, R. C. J. Miller, and R. A. J. Warren.** 1991. Domains in microbial beta-1,4-glycanases: sequence conservation, function, and enzyme families. Microbiol. Rev. **55**:303–315.
9. **Gilkes, N. R., E. Jervis, B. Henrissat, B. Tekant, R. C. J. Miller, R. A. J. Warren, and D. G. Kilburn.** 1992. The adsorption of a bacterial cellulase and its two isolated domains to crystalline cellulose. J. Biol. Chem. **267**:6743–6749.
10. **Hansen, C. K.** 1992. Fibronectin type III-like sequences and a new domain type in prokaryotic depolymerases with insoluble substrates. FEBS Lett. **305**:91–96.
11. **Innis, M. A., and D. H. Gelfand.** 1990. Optimization of PCRs, p. 3–12. *In* M. A. Innis, D. H. Gelfand, J. J. Sninsky, and T. J. White (ed.), PCR protocols: a guide to methods and applications. Academic Press, Inc., San Diego, Calif.
12. **Kellett, L. E., D. M. Poole, L. M. A. Ferreira, A. J. Durrant, G. P. Hazlewood, and H. J. Gilbert.** 1990. Xylanase B and an arabinofuranosidase from *Pseudomonas flourescens* subsp. *cellulosa* contain identical cellulose-binding domains and are encoded by adjacent genes. Biochem. J. **272**:369–376.
13. **Klyosov, A. A.** 1988. Cellulases of the third generation, p. 87–99. *In* J.-P. Aubert, P. Beguin, and J. Millet (ed.), Biochemistry and genetics of cellulose degradation. Academic Press Ltd., London.
14. **Klyosov, A. A.** 1990. Trends in biochemistry and enzymology of cellulose degradation. Biochemistry **29**:10577–10585.
15. **Lamed, R., and E. A. Bayer.** 1988. The cellulosome concept, p. 101–117. *In* J.-P. Aubert, P. Beguin, and J. Millet (ed.), Biochemistry and genetics of cellulose degradation. Academic Press Ltd., London.
16. **Mayer, F., M. P. Coughlan, Y. Mori, and L. G. Ljungdahl.** 1987. Macromolecular organization of the cellulolytic enzyme complex of *Clostridium thermocellum* as revealed by electron microscopy. Appl. Environ. Microbiol. **53**:2785–2792.
17. **Poole, D. M., E. Morag, R. Lamed, E. A. Bayer, G. P. Hazlewood, and H. J. Gilbert.** 1992. Identification of the cellulose-binding domain of the cellulosome subunit S1 from *Clostridium thermocellum* YS. FEMS Microbiol. Lett. **99**:181–186.
18. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
19. **Segel, I. H.** 1975. Enzyme kinetics, p. 18–97. Wiley Interscience, New York.
20. **Shoseyov, O., and R. H. Doi.** 1990. Essential 170-kDa subunit for degradation of crystalline cellulose by *Clostridium cellulovorans* cellulase. Proc. Natl. Acad. Sci. USA **87**:2192–2195.
21. **Shoseyov, O., M. Takagi, M. A. Goldstein, and R. H. Doi.** 1992. Primary sequence analysis of *Clostridium cellulovorans* cellulose binding protein A. Proc. Natl. Acad. Sci. USA **89**:3483–3487.
22. **Sleat, R., R. A. Mah, and R. Robinson.** 1984. Isolation and characterization of an anaerobic, cellulolytic bacterium, *Clostridium cellulovorans* sp. nov. Appl. Environ. Microbiol. **48**:88–93.
23. **Studier, F. W., and B. A. Moffat.** 1986. Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes. J. Mol. Biol. **189**:113–130.
24. **Szmelcman, S., M. Schwartz, T. J. Silhavy, and W. Boos.** 1976. Maltose transport in *E. coli* K12. Eur. J. Biochem. **65**:13–19.
25. **Weimer, P. J., A. D. French, and T. A. Calamari.** 1991. Differential fermentation of cellulose allomorphs by ruminal cellulolytic bacteria. Appl. Environ. Microbiol. **57**:3101–3106.
26. **Wilkinson, F.** 1980. Chemical kinetics and reaction systems, p. 52–58. Van Nostrand Reinhold Co., New York.
27. **Young, R. A., and R. M. Rowell.** 1986. Cellulose structure and biosynthesis, p. 3–50. *In* R. A. Young, and R. M. Rowell, Cellulose: structure, modification, and hydrolysis. Wiley Interscience, New York.